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ORIGINAL PAPER

Mixture of sugar and povidone–iodine stimulates wound healing by activating keratinocytes and fibroblast functions

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Abstract The topical application of a mixture of sugar and povidone–iodine (PI) has been reported to accelerate the healing of cutaneous wounds and ulcers by promoting re-epithelialization and granulation tissue formation as well as having an anti-microbial effect. To clarify the mechanisms accounting for the efficacy of a 70% sugar and 3% PI paste (U-PASTA™) (SP), various keratinocytes and fibroblasts functions, including proliferation, collagen synthesis, integrin expression, and cytokine and proteinase secretions in the presence of SP were investigated. Cultured human keratinocytes and fibroblasts were treated with various concentrations of SP, SU and PI. The secretion of urokinase-type plasminogen activator (u-PA), transforming growth factor (TGF)-α and interleukin-1α from keratinocytes, was detected by ELISA. Collagen synthesis of fibroblasts was examined by means of detecting proline uptake. Furthermore, integrin expressions of these cells were analyzed using a flow cytometer. SP and PI increased intra-cellular u-PA of keratinocytes and stimulated the secretion of u-PA and TGF-α. Sugar accelerated the extra-cellular u-PA level only. Both SP and sugar increased the collagen synthesis of fibroblasts. SP and PI also remarkably induced the expressions of extra-cellular matrix receptor integrins, α1, α2, α3, α4, α5 and β1, on the surface of keratinocytes and fibroblasts. SP, the mixture of sugar and PI, is likely to act on wounds not only as an antibiotic agent, but also as a modulator for keratinocytes and fibroblasts.

Keywords Topical agent · Cytokine · Integrin · Collagen · Plasminogen activator

Abbreviations

IL-1α	Interleukin-1α
PI	Povidone–iodine;
SU	A paste comprising 70% sugar
SP	A paste comprising 70% sugar and 3% PI
TGF-α	Transforming growth factor-α
TGF-β	Transforming growth factor-β
u-PA	Urokinase-type plasminogen activator

Introduction

Sugar and related products from various natural sources have been used to promote wound healing [16]. Povidone–iodine (PI), a complex of iodine and polyvinylpyrrolidone, is a common anti-microbial agent [22] and has been used as a surgical scrub or a skin cleanser in various forms. The use of PI as a topical healing-stimulatory agent has been limited because of its toxicity [3, 5, 33]. Knutson et al. [19] have reported in a clinical study that a compound consisting of 70–80% sugar and PI solution remarkably enhanced

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healing and reduced bacterial contamination in a wide variety of wounds, burns and ulcers. Similarly, the topical application of a mixture of sugar and PI successfully treated diabetic and burn ulcers [2, 29]. A paste consisting of 70% sugar and 3% PI has been commercially available in Japan (U-PASTA™, Kowa company Ltd, Nagoya, Japan) for some time. In Europe and North America, a paste not commercially available, consisting of sugar and PI, is commonly used on chronic wounds. Cadexomer-iodine is the commercially available counterpart [4, 35]. It is reported that U-PASTA is clinically effective in promoting rapid wound healing and in reducing bacterial contamination [23, 24, 28].

During re-epithelialization and granulation tissue formation, keratinocytes and fibroblasts migrate, proliferate and synthesize the extra-cellular matrix. Cytokines released from these cells further these activities in both types of cell [30]. The urokinase-type plasminogen activator (u-PA) is known to play an important role in the migration of keratinocytes and the activation of latent collagenase and transforming growth factor (TGF)-α [10, 14, 25]. The expression of integrins is also associated with the ability of keratinocytes and fibroblasts to attach to the extra-cellular matrix and migrate into the wound site [7]. In the process of migrating over the surface of injured skin, keratinocytes are known to express primarily the α5β1 and αvβ6 fibronectin/tenascin receptors, the αvβ5 vitronectin receptor and the α2β1 collagen receptors [6, 9, 15, 20, 26]. Fibroblasts also use β1 integrins, such as αvβ1, α4β1, α5β1, for attachment to fibronectin, αvβ3 and αvβ5 for attachment to vitronectin [12], and α2 for attachment to collagen [17]. TGF-β1 up-regulates the expression of α5β1, αvβ5 and α2β1, and down-regulates α3β1 in keratinocytes in spite of inhibiting their proliferation [34]. TGF-β1 is also known to stimulate the synthesis of extra-cellular matrix from fibroblasts and to be the most potent inducer of procollagen [8].

To clarify the pharmacological effect of sugar and PI on wound healing, we have studied in this paper various keratinocyte and fibroblast functions in the presence of SP, including proliferation, collagen synthesis, integrin expression, and cytokine and proteinase secretions.

Materials and methods

Reagents

A commercially available paste (SP) consisting of 70% sugar (sucrose) and 3% PI (US patent 4844898, U-PASTA), manufactured by Kowa Company Ltd under aseptic conditions, was used. SP was developed to fill a need for a substance that was chemically stable, physically homogeneous and spreadable, unlike a simple mixture of sugar and PI. SP contains a water-soluble base including polyethylene glycol 400, glycerin and water, in addition to sugar and PI. For the following experiment, a paste containing 70% sugar and a soluble base (SU) was prepared by Kowa Company Ltd (Nagoya, Japan). The PI was of the same grade as that of the Japanese Pharmacopoeia, and the other chemicals were of the highest grade commercially available. The media containing the SP, SU and PI were sterilized by filtration just before they were added to the cultured cells.

Cell cultures

Normal human keratinocytes from neonatal foreskins and normal human fibroblasts from breast skin were purchased from Kronetics (San Diego, CA, USA). They were grown in the keratinocyte growth medium (Kurabo Co., Osaka, Japan) and Dulbecco's modified Eagle's medium (Nikken Biomedical Lab., Kyoto, Japan) with 10% fetal calf serum, respectively, as described in a previous report [21, 31].

Proliferation assays

Keratinocytes and fibroblasts were seeded at a density of 3,000 and 5,000 cells/cm^2, respectively, on 24-well plates (Corning, New York, NY, USA) in 0.5 ml growth medium. Twenty-four hours after seeding, the medium was changed to 1.0 ml of the fresh growth medium containing various concentrations of SP, SU or PI. After a 48-h culture, the cells were incubated with [^{3}H]-thymidine (>10 Ci/nmol, NEN, Boston, MA, USA) for 5 h at a final concentration of 1 μCi/well, then washed three times with Hanks' balanced salt solution and solubilized with 150 μl of 0.1% sodium dodecyl sulfate. The radioactivity was measured in a liquid scintillation analyzer (Tri-Carb 1500, Packard, Meriden, CT, USA) after the addition of Emulsifier Scintillator Plus (Packard, Meriden, CT, USA).

Measurement of cytokines and u-PA

The keratinocyte-conditioned medium was collected after a 48-h culture. Cell lysates were prepared with a sonicator (UD-200, Tomy, Tokyo, Japan). Concentrations of cytokines and u-PA were measured using ELISA (TMB peroxidase substrate kit, Bio-Rad Laboratories, Hercules, CA, USA). Specific antibodies for TGF-α (GH Healthcare, Buckinghamshire, UK),

interleukin-1 (IL-1)-α (GH Healthcare), TGF-β1 (GH Healthcare) and u-PA (Fuji Pharmacy, Toyama, Japan) were used for this system according to the manufacturer's instructions. The DNA concentration of cell lysates was determined by Kissane's method [18] with calf thymus DNA (Sigma, St Louis, MO, USA) as a standard.

Flow cytometry

Integrins expressed on cell surfaces were incubated with integrin-specific antibodies, rabbit polyclonal antibodies against human α1, α2, α3, α4 and α5 integrins from Chemicon International (Temecula, CA, USA), a mouse monoclonal antibody against human β1 integrin (DF5) from Biohit (Helsinki, Finland) and isotype-matched immunoglobulins as a negative control for 1 h on ice. After washing twice with PBS, the cells were incubated with FITC-labeled goat anti-rabbit IgG $(Fab')_2$ (Leinco Technologies, Ballwin, MO, USA) or goat anti-mouse IgG$(Fab')_2$ (Leinco Technologies) for 30 min on ice. After washing and fixing with 0.5% formalin, the cells were analyzed by EPICS XL (Coulter, Hialeah, FL, USA) equipped with IsoFlow (Coulter). The results were presented as parameter histograms.

Collagen synthesis

Fibroblasts were seeded on 96-well plates (Corning) at a density of 4,000 cells/well in 0.2 ml of growth medium. Twenty-four hours after seeding, the medium was changed to 0.2 ml of medium containing various concentrations of SP or SU, and 1 mCi of [^{3}H]-proline (99 Ci/mmol, GH Healthcare). After a 48-h incubation, [^{3}H]-proline incorporation into pepsin-resistant, salt-precipitable, extra-cellular collagen was determined by means of a previously described method [32]. The results were expressed as dpm of [^{3}H]-collagen per well.

Statistical analysis

The data obtained were compared with other groups using the Student's paired *t*-test

Results

Dose-dependent cellular toxicity of SP, SU and PI on keratinocytes and fibroblasts

The dose response of SP, SU and PI on the proliferation of keratinocytes and fibroblasts was examined by using [^{3}H]-thymidine uptake after the 48-h culture. As shown in Fig. 1, SP and SU did not inhibit the proliferation of keratinocytes at concentrations of less than 10 mg/ml, and at concentrations of less than 1 mg/ml for fibroblasts. The decreased ratio of thymidine uptake in the keratinocytes incubated with 10 mg/ml of SP and SU was about 40 and 10%, compared to those with growth media without SP and SU, respectively (Fig. 1a). The same extent of proliferation inhibition was observed after the incubation of fibroblasts with SP and SU (Fig. 2b). The proliferation of keratinocytes and fibroblasts was not inhibited by 0.1 and 0.01 mg/ml

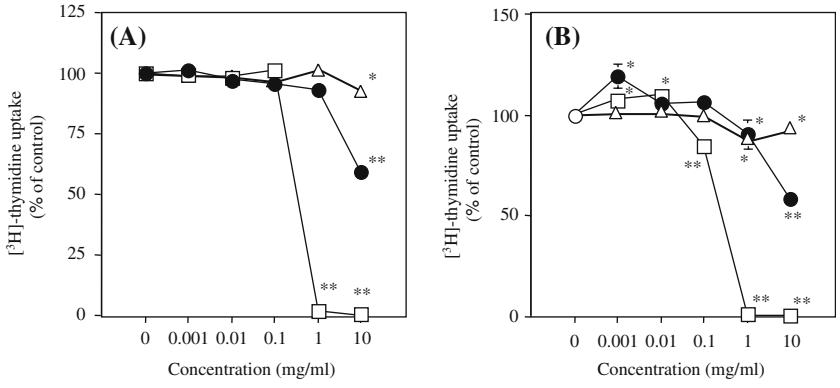

Fig. 1 Dose-dependent cellular toxicity of SP, SU and PI on keratinocytes and fibroblasts. Keratinocytes (**a**) and fibroblasts (**b**) cultured for 24 h were transferred to a medium containing the indicated concentration of a paste consisting of 70% sugar and 3% povidone–iodine (*SP*, *closed circle*) or a paste consisting of 70% sugar (*SU*, *triangle*) or povidone–iodine (*PI*, *square*). Next, following a 48-h culture, the cells were incubated with [^{3}H]-thymidine for 5 h, then washed and solubilized. Solubilized radioactivity was measured in a liquid scintillation. The results are a compilation of three experiments repeated three times each. Data are expressed as the mean ± standard error of means (*SEM*) of quadruplicate values. $*P < 0.05$ and $**P < 0.01$ compared with the control groups

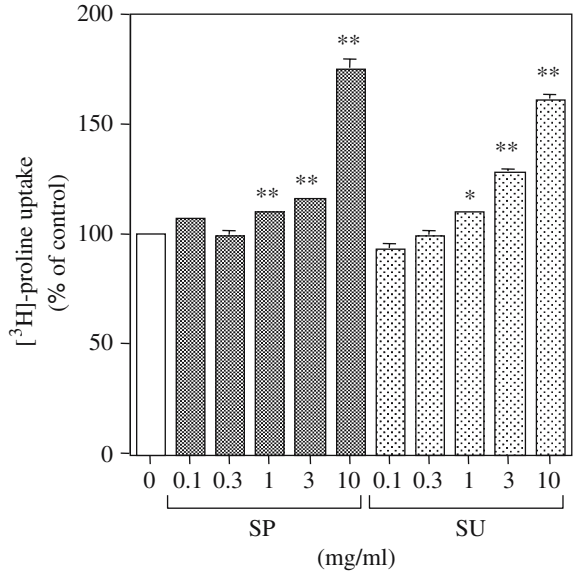

Fig. 2 SP promoted collagen synthesis from fibroblasts. Fibroblasts cultured for 24 h were transferred to a medium containing the indicated concentration of SP or SU, and [^{3}H]-proline. After the next 48-h culture, [^{3}H]-proline incorporated into pepsin-resistant, salt-precipitable extra-cellular collagen was extracted and then solubilized. Solubilized radioactivity was measured in a liquid scintillation. Data are expressed as the mean + SEM of triplicate values from two experiments. $^*P < 0.05$ and $^{**}P < 0.01$ compared with the control groups

of PI, respectively, although it was completely inhibited by 1 mg/ml of PI.

SP stimulated u-PA production from keratinocytes

The effects of SP, SU and PI on intra- and extra-cellular levels of u-PA from keratinocytes were examined using ELISA assays after a 48-h culture. As shown in Table 1, SP significantly stimulated an increase in intra- and extra-cellular u-PA at 1 mg/ml concentrations at the efficacy rates of 23 and 78%, respectively. SU also promoted keratinocyte secretion of u-PA at 1 and 10 mg/ml by 23 and 38%, respectively. On the contrary, PI significantly suppressed extra- and intra-cellular u-PA at 0.3 and 0.003 mg/ml, respectively. Extra-cellular levels of u-PA following treatment with 10 mg/ml of SP and 0.3 mg/ml of PI decreased, while their intra-cellular levels increased.

SP stimulated the secretion of TGF-α from keratinocytes

The effects of SP, SU and PI on the secretion of TGF-α and IL-1α by keratinocytes were examined using ELISA assays after a 48-h culture. SP promoted the

Table 1 SP promoted secretion of u-PA by keratinocytes and increase in intra-cellular levels

Chemicals (mg/ml)	Medium (u/ml)	Cell (u/μg DNA)
Control	5.817 ± 0.088	0.436 ± 0.015
SP 0.1	6.738 ± 0.212**	0.442 ± 0.031
SP 1	7.187 ± 0.147**	0.777 ± 0.051**
SP 10	5.961 ± 0.165	0.877 ± 0.070**
SU 0.1	5.453 ± 0.691	0.439 ± 0.034
SU 1	7.163 ± 0.099**	0.486 ± 0.071
SU 10	8.051 ± 0.066**	0.555 ± 0.060
PI 0.003	5.677 ± 0.355	0.381 ± 0.011**
PI 0.03	5.549 ± 0.159	0.675 ± 0.018**
PI 0.3	3.817 ± 0.148**	0.534 ± 0.030*

Each value represents the mean ± SEM of values from quadruplicate

$^*P < 0.05$ and $^{**}P < 0.01$ compared with the control groups

secretion of TGF-α from keratinocytes at the concentrations of 0.1, 1 and 10 mg/ml at the efficacy rates of 11, 47 and 9%, respectively. SU promoted secretion only by 9% at a concentration of 10 mg/ml (Table 2). PI promoted TGF-α secretion from keratinocytes at 0.003, 0.03 and 0.3 mg/ml by 16, 38 and 26%, respectively. However, most of the concentrations of SP, SU and PI used did not affect IL-1α secretion from keratinocytes. In fact, PI at a concentration of 0.3 mg/ml decreased secretion. TGF-β level in the conditioned medium of keratinocytes was less than measurable and therefore not detected.

SP promoted collagen synthesis from fibroblasts

The effect of SP and SU on collagen synthesis by fibroblasts was examined by measuring [^{3}H]-proline incorporation during a 48-h culture. As shown in Fig. 2, the

Table 2 SP promoted secretion of TGF-α and IL-1α by keratinocytes

Chemicals (mg/ml)	TGFα (pg/ml)	IL-1α (pg/ml)
Control	80.57 ± 1.85	11.06 ± 1.24
SP 0.1	96.03 ± 1.34**	10.99 ± 0.41
SP 1	118.50 ± 1.32**	10.56 ± 0.24
SP 10	88.53 ± 2.03*	11.71 ± 0.51
SU 0.1	82.21 ± 0.90	10.35 ± 0.34
SU 1	84.08 ± 0.97	9.71 ± 0.49
SU 10	88.53 ± 2.99*	9.99 ± 1.02
PI 0.003	94.16 ± 0.88**	9.78 ± 0.34
PI 0.03	111.50 ± 1.55**	9.49 ± 0.34
PI 0.3	102.14 ± 1.38**	8.20 ± 0.31*

Each value represents the mean ± SEM of values from quadruplicate

$^*P < 0.05$ and $^{**}P < 0.01$ compared with the control groups

promoting effect of proline uptake was observed in a dose-dependent manner. At 1.0 mg/ml, SP promoted collagen synthesis by fibroblasts; at 10 mg/ml, the rate of collagen synthesis had increased remarkably by about 80%. SU was also found to have an effect similar to that of SP.

SP enhanced integrin expressions on the cell surface of keratinocytes and fibroblasts

The effects of SP, SU and PI on the expressions of cell surface integrin receptors in keratinocytes (Fig. 3) and fibroblasts (Fig. 4) were examined by an immuno-staining method following a 48-h culture. As shown in Figs. 3 and 4, at a concentration of 10 mg/ml, SP promoted the expression of integrins such as α1, α2, α3, α4, α5 and β1 in both types of cell. However, at a concentration of 10 mg/ml, SU either had only a slight effect or none at all. At a concentration of 0.3 mg/ml, PI promoted α1, α2, α3, α4, α5 integrin expressions in both types of cell, though its effect was weaker than that of SP; PI had little or no effect in β1 integrin expression in either the keratinocytes or the fibroblasts. Neither SP at a concentration of 1 mg/ml nor PI at a concentration of 0.03 mg/ml affected integrin expression in either type of cell (data not shown).

Discussion

The topical application of the mixture of sugar and PI has been reported in clinical trials to be effective in promoting healing in various cutaneous wounds, including burns and chronic ulcers [2, 19, 23, 24, 28, 29]. The efficacy for wound healing of the sugar and PI mixture has been shown to be different from that of sugar alone. Some of the mechanisms thought to underlie sugar-promoted wound healing are the osmotic effect preventing bacterial growth [1] and the acceleration of granulation tissue formation partly by the mechanical cleansing of necrotic tissue in wounds [13]. The mixture of sugar and PI is also thought to have an antimicrobial/antibiotic effect, sugar itself has a hygroscopic effect, which reduces edema in wounds while simultaneously carrying PI deep into the wound or ulcer, thereby eliminating deeper contamination [19]. Although both SP and SU promoted re-epithelialization and granulation tissue formation in normal full-thickness wounds in rats [11], the difference in efficacy between SP and SU has also been recognized in other animal studies; the topical application of SP on full-thickness wounds of diabetic mice promoted granulation tissue formation, but SU did not [27]. Moreover, SP application on normal full-thickness wounds in rabbits promoted re-epithelialization and granulation tissue formation, but SU promoted re-epithelialization only [27].

In the present study, we demonstrated that SP and PI up-regulated intra- and extra-cellular u-PA levels and TGF-α production in keratinocytes, and the expression of extra-cellular matrix receptor integrins such as α1, α2, α3, α4, α5 and β1 in both keratinocytes and fibroblasts. Furthermore, we demonstrated that SP and SU accelerated collagen synthesis in fibroblasts. These results suggest that SP influences the migration of keratinocytes and fibroblasts by the pharmaceutical effects of PI on these cells, whereas SU mainly stimulated the increase of granulation tissue. The u-PA is known to play an important role in the migration of keratinocytes and the activation of latent collagenase and TGF-α [10, 14, 25]. In the present study, the extra-cellular level of u-PA decreased as a result of treatment with 10 mg/ml of SP and 0.3 mg/ml of PI, while their intra-cellular levels were increased (Table 1). This may be due to the loss of cells resulting from the cytotoxic effect of higher concentration of these reagents. SU was the best stimulator of u-PA because its cytotoxic effect was minimal. However, SU did not induce as much secretion of TGF-α as SP and PI, although both 10 mg/ml of SU and 10 mg/ml of SP did (Table 2). It is possible that PI stimulates TGF-α secretion via integrin signals more effectively than the up-regulation of u-PA. IL-1α, one of the major inflammatory cytokines, decreased only after the addition of 0.3 mg/ml of PI (Table 2), probably as a result of the cytotoxic effect of PI. SP, SU and PI did not promote the production of this cytokine from keratinocytes (Table 2), suggesting that these reagents had the effect of specifically promoting TGF-α. The cellular toxicity of PI for fibroblasts is well known; proliferation is progressively retarded by 0.01 and 0.025% PI solutions, and completely inhibited by 0.1 and 1% PI solutions [3]. In the present study, 1 mg/ml (0.1%) and 0.1 mg/ml (0.01%) PI solutions inhibited the proliferation of keratinocytes and fibroblasts, respectively, and 10 mg/ml of SP containing 0.03% PI inhibited the proliferation of both types of cell by approximately 40%. This suggests that fibroblasts are more vulnerable to the cytotoxicity of PI than keratinocytes are. On the other hand, at this concentration, SP induced u-PA and TGF-α production and up-regulated integrins in keratinocytes and fibroblasts. Although the toxic effects of PI are apparent in experiments involving cell culture systems, the ameliorative effects of SP on wound healing have been proven in animal models and clinical practice. Since SU acceler-

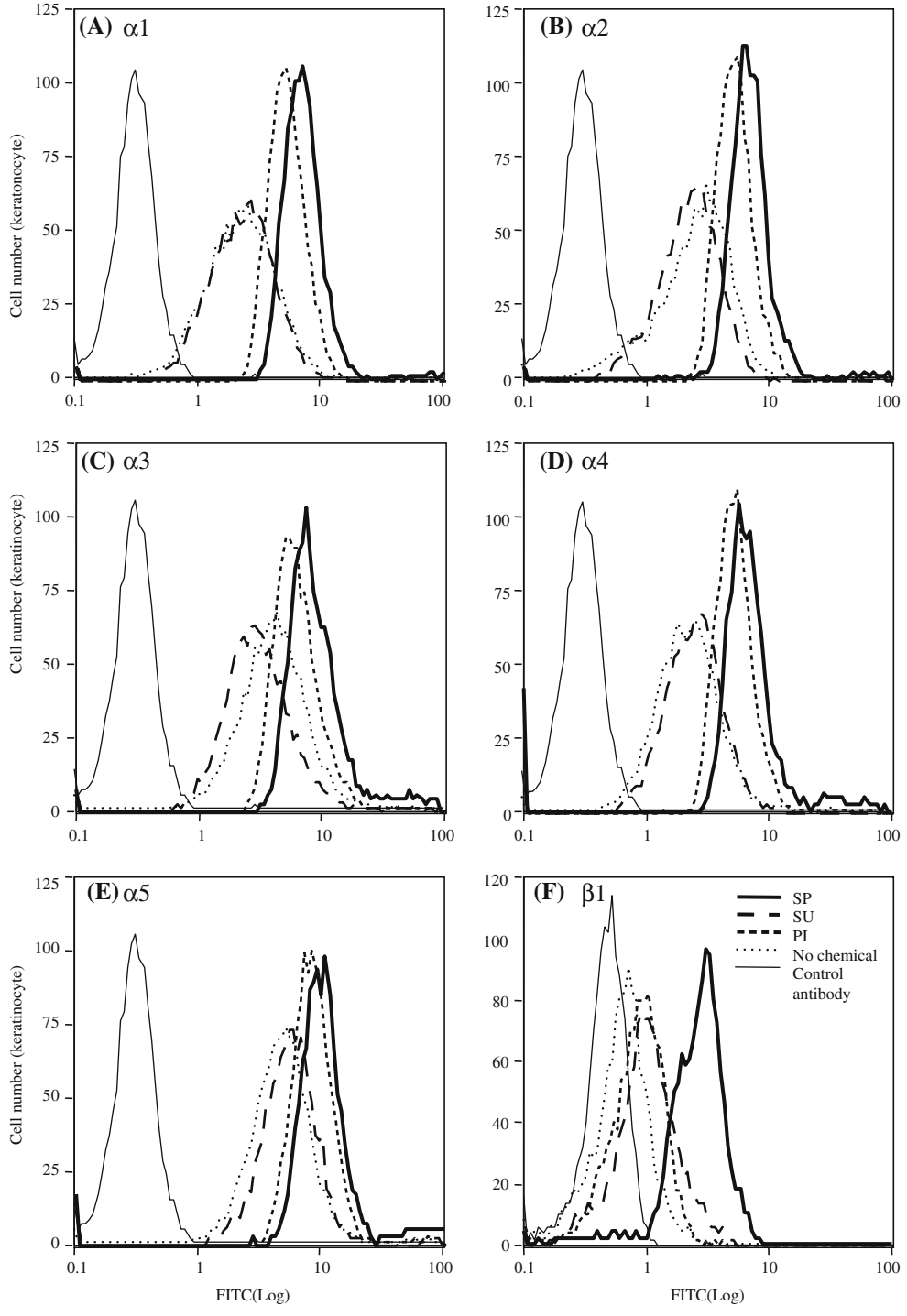

Fig. 3 SP enhanced integrin expressions on the cell surface of keratinocytes. Keratinocytes cultured for 24 h were transferred to a medium containing SP (10 mg/ml), SU (10 mg/ml), PI (0.3 mg/ml) or no reagent. After the next 48-h culture, integrins expressed on the cell surface were stained with integrin-specific antibodies against α1 (**a**), α2 (**b**), α3 (**c**), α4 (**d**), α5 (**e**) and β1 (**f**) integrins, and negative control antibody (*thin solid line*), further stained with FITC-labeled antibody and then analyzed on EPICS XL. Each histogram is representative of experiments repeated three times

ated collagen synthesis in fibroblasts, as shown in the present study, it is possible that adequate concentrations of sugar in clinical applications may induce the secretion of some peptides or proteins from fibroblasts, which may in turn neutralize the active free iodine released from SP.

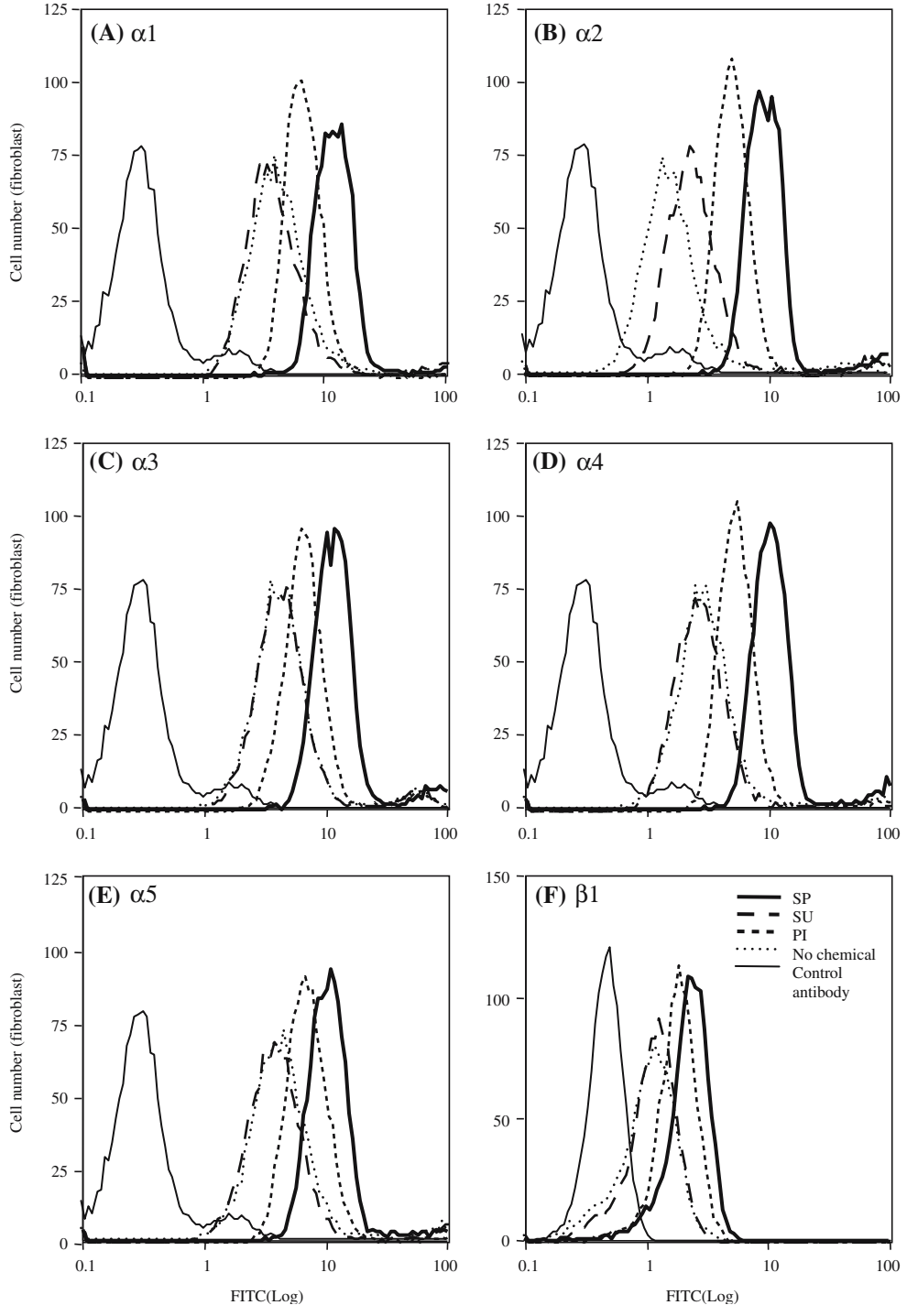

Fig. 4 SP enhanced integrin expressions on the cell surface of fibroblasts. Fibroblasts cultured for 24 h were transferred to a medium containing SP (10 mg/ml), SU (10 mg/ml), PI (0.3 mg/ml) or no reagent. After the next 48-h culture, integrins expressed on the cell surface were stained with integrin-specific antibodies against α1 (**a**), α2 (**b**), α3 (**c**), α4 (**d**), α5 (**e**) and β1 (**f**) integrins and control antibody (*thin solid line*), then stained with FITC-labeled antibody, and analyzed on EPICS XL. Each histogram is representative of the experiments repeated three times

In the present study, we demonstrated that a mixture consisting of sugar and PI activated the functions of keratinocytes and fibroblasts more effectively than sugar alone, and has the potential to be a powerful ameliorative agent in the promotion of wound healing. These results further indicate that SP likely acts on

wounds not only as an antibiotic agent, but also as a modulator for keratinocytes and fibroblasts.

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